A VITAMIN D-DEPENDENT CALCIUM-BINDING PROTEIN IN RAT SKIN

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1. Introduction

Calcium binding protein (CaBP) is one of the known molecular expressions of the hormonal action of the calciferols (vitamin D) on intestine, especially in birds [1,2] and in rats [3,4]. CaBPs similar to or different from intestinal CaBP have been isolated from several tissues: brain [5], bone [6], kidney [7,8], uterus [9], parotid gland [10], parathyroid glands [11,12], and placenta [13]. Some, but not all, of these CaBPs have been shown to be vitamin D-dependent, i.e., their amount, low or undetectable in D-deficient animals, increases significantly following vitamin D administration.

The skin is a unique organ for the synthesis, storage, and release of vitamin D [14,15]. The present study was undertaken in order to see whether CaBP is also present in this tissue. The results indicate that a vitamin D-dependent CaBP of a molecular weight (9000 ± 2000) similar to that of rat intestinal CaBP (11 000 ± 2000), but immunologically different from the latter, is present in rat skin.

2. Materials and methods

Male adult Sprangley albino rats (Charles River®, Elbeuf) ~200 g, raised on a diet normal in calcium (0.7%) and in phosphorus (0.7%), containing 4 IU vitamin D_3/g diet were used. After removal of hair a skin sample (3 × 3 cm) from the dorsal region of each animal was taken under ether anesthesia. The skin samples were weighed, freezed by immersion into liquid nitrogen, minced (Moulinex type 302, Alençon) and homogenized in Tris buffer using a blender (homogénéiseur Baudard, Paris). The Tris buffer contained 1.37×10^{-2} M Tris–HCl, 0.119 M NaCl, 4.74×10^{-3} M KCl (pH 7.4). Homogenization and all subsequent steps of extraction were carried out at 0–4°C. The homogenate was centrifuged for 25 min at 3000 × *g* and the supernate was decanted. This low speed centrifugation was chosen in view of the possibility that the CaBP, if present in the skin, could be localized in cell membrane, in the cytosol, in mitochondria and/or in other intracellular organelles fractions as shown for chick and rat intestinal CaBP [3,16,17]. The supernate recovered after centrifugation was lyophilized, redissolved in 4–5 ml elution buffer (0.02 M ammonium acetate 0.02% sodium azide (pH 7.4)) and chromatographed on a 2.5 × 85 cm Sephadex G-75 column. The flowrate was 35 drops/min, and 5 ml fractions were collected. The fractions were analyzed for protein [18], and for calcium-binding activity by the Chelex 100 competitive binding assay [19].

Further purification of the CaBP was obtained using DEAE-Sephadex A-25 ion exchange chromatography in a buffer containing 0.02 imidazole, 0.02 M NaCl, 1 mM EDTA (pH 7.2) as in [20,21], and a linear 0.20–0.70 M NaCl gradient. The proteins were subsequently desalted by application of the eluate to a Biogel P6 column equilibrated with ammonium acetate buffer. Void volume fractions were tested for calcium-binding activity. To check the purity of the protein obtained at each step of the procedure, polyacrylamide gel electrophoresis was used according to [22] in the presence of 0.06 mM EDTA.

Molecular weight of skin CaBP was estimated by means of the calibrated Sephadex G-75 column [17].

Measurements of CaBP were performed using an electroimmunodiffusion method. For this purpose antiserum to purified skin CaBP (anti-CaBP) was raised by injecting intradermally into several sites of the back of three 5 month white rabbits 150 μg

CaBP emulsified in Freund's complete adjuvant. This initial dose was followed by a second injection after 4 weeks. On day 49, blood was withdrawn by veinous puncture, the presence of anti-CaBP, its specificity and cross reactivity were tested using Outcherlony's double immunodiffusion technique [23]. The anti-CaBP thus obtained was included in a buffered agarose solution (1% agarose, 0.02 ionic strength barbital buffer (pH 8.6)) at final conc. 8%. This solution was distributed as a uniform layer over a 5 × 10 cm glass plate. CaBP solutions (5 μl) were placed in 2.5 mm diam. holes punched in the gel 0.5 cm from one short edge. Electrophoresis was carried out for 2 h at 200 V. Precipitin lines were made visible by staining with a 1% solution of amide black 10 B [21]. The height of each 'rocket' was measured and used as an index of CaBP concentrations. A standard curve was obtained with purified skin CaBP. Appropriate dilutions in normal saline of the skin crude extract, obtained after homogenization and centrifugation, were used for the measurements of individual skin CaBP. With this technique the interassay variation is 3.4% and the detection limit 0.05 μg CaBP/mg protein.

In an attempt to localize the cellular distribution of the skin CaBP the amount and the immunoreactivity of the protein obtained in the 3000 × *g* supernatant were compared to those of the protein obtained in a 100 000 × *g* supernatant.

For the study of the vitamin D-dependence of skin CaBP male 21 day rats were fed a diet containing no vitamin D and a normal (0.7%) or low content (0.02%) of calcium. After 4 weeks of this diet half of the animals were given by oral intubation 1 μg vitamin D_2/day for 3 days. The amount of CaBP in each animal's skin was measured and the result (mean ± SE) was compared to that observed in the vitamin D-deprived control group. The concentration of calcium in serum was determined individually with the semiautomatic complexometric technique using a titrator (Marius Calcium Titrator, Amsterdam). Student's *t*-test was used for statistical analysis.

3. Results

When a 3000 × *g* supernatant of rat skin homogenate was chromatographed on Sephadex G-75 and the ^{45}Ca-binding activity of the different fractions studied, 3 peaks were observed (fig.1): a chromatographic pattern similar to the one reported for intestinal extract [4,21]. Peak I appeared at the void volume. Proteins of peak II showed the highest calcium-binding activity, and the maximum activity appeared for an elution volume where $V_e/V_o = 2.0$. On ion-exchange DEAE A-25 Sephadex column CaBP from peak II was eluted in the higher anionic form (EDTA 1 mM) at 0.14 M NaCl; in the same system, rat intestinal CaBP elutes at 0.12 M NaCl (data not shown).

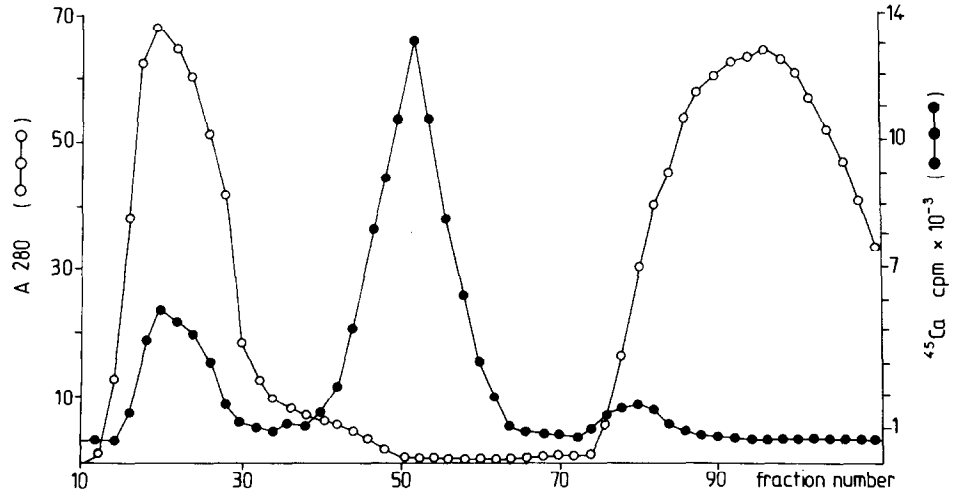

Fig.1. Sephadex G-75 filtration of a 3000 × *g* supernatant of rat skin. Protein concentration (open circles) and calcium binding activity (closed circles) in the fractions eluted from Sephadex G-75 column. These results are those of the soluble heat-stable proteins from the 3000 × *g* supernatant of rat skin extract.

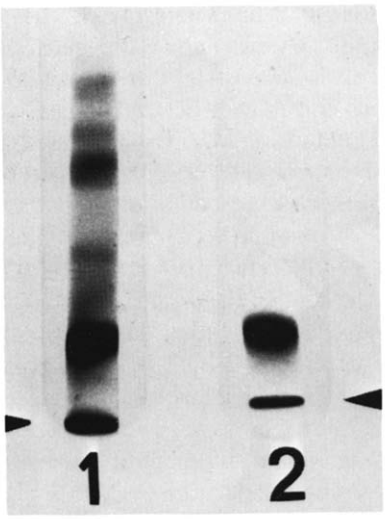

Fig.2. Acrylamide gel electrophoresis (pH 8.6) of aliquots of skin material from two stages of purification: (1) peak II (60 μg protein) from Sephadex gel filtration; (2) purified CaBP (20 μg protein) eluted from DEAE A25 chromatography. Samples were placed on the top of the gels and 3 mA/tube were applied for 90 min. Migration was toward the anode (bottom) in presence of EDTA–Tris glycine buffer. The arrows indicate the localization of the tracking dye (bromophenol blue).

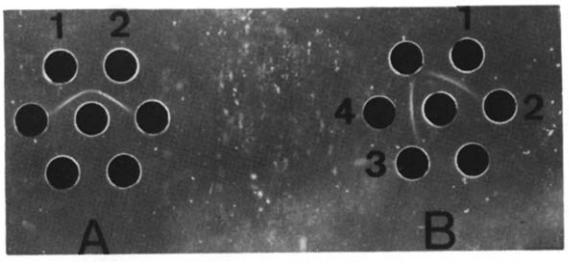

Fig.3. Ouchterlony double immunodiffusion: The center wells in A and B contain the antiserum to skin CaBP; (A) the outer well 1 contains the 3000 × *g* supernatant from skin extract, the outer well 2 the purified skin CaBP; (B) the outer well 1 contains purified skin CaBP, the one labeled 2 purified intestinal CaBP, the wells marked 3 and 4 contain supernatant from crude extracts of intestinal mucosa and skin, respectively.

Fig.2 shows the electrophoretic pattern of the pooled fractions of skin CaBP (peak II) after Sephadex G-75, and the purification achieved after DEAE–Sephadex chromatography. The molecular weight of this purified skin CaBP, estimated using a calibrated column is 9000 ± 2000, a value not different from the one found for rat intestinal CaBP, i.e., 11 000 ± 2000 [17]. And the comparison of the staining pattern of purified intestinal and skin CaBPs showed the similarity of the anionic properties of both CaBPs in presence of EDTA (R_F 0.80 and 0.84, respectively).

The specificity of the rabbit antiserum was analyzed by double immunodiffusion [23]. The purity of the skin protein used to raise the antiserum was indicated by the presence of a single precipitin line when the skin extract was reacted against the antiserum (fig.3A1,B4). No precipitation lines were obtained when the purified skin CaBP was incubated against non-immunized rabbit serum. Specificity of the rabbit antiserum to skin CaBP was shown by the total coalescence of crude skin extract and purified skin CaBP (fig.3A1,A2). Rat intestinal CaBP, purified from an aqueous extract obtained after a 100 000 × *g* centrifugation, did not crossreact with the antiserum to skin CaBP (fig.3B2). Conversely, cross-immunoreactions could not be detected when rat intestinal antiserum was used against skin CaBP (data not shown).

The immunoreactivity of the skin CaBP obtained from the fractionation of the 3000 × *g* supernatant and that obtained from the 100 000 × *g* supernatant were compared. The results showed that the 100 000 × *g* supernatant CaBP reacted to the antiserum in a manner identical to that of the 3000 × *g* supernatant. And, once again, no cross-immunoreaction against rat intestinal CaBP antiserum could be detected neither with the 3000 × *g* supernatant nor with the 100 000 × *g* supernatant skin CaBP.

The amount of CaBP present in the skin of normal rats, evaluated by electroimmunodiffusion assay [24] was found to be 27.1 ± 2.2 μg/mg total protein. When the amounts of CaBP found in the 3000 × *g* supernatant, and in the 100 000 × *g* supernatant were compared, it appeared that the 100 000 × *g* supernatant contained 24.5 μg/mg protein, i.e., 90.5% of the amount found in the 3000 × *g* supernate.

The effects of different dietary intakes of vitamin D and of calcium are shown in table 1. A diet of 4 weeks without vitamin D induced in rats a significant decrease in serum calcium (8.0 ± 0.3 versus 10.3 ± 0.1, $p < 0.001$). In these rats the amount of CaBP in the 3000 × *g* supernatant of skin decreased to 15.9 ± 1.2 ($p < 0.001$). In rats fed a diet without vitamin D and low in calcium severe hypocalcemia was observed (5.5 ± 0.1, $p < 0.001$). In this group a considerable amount of CaBP was still present in

Table 1
Effects of vitamin D status and different dietary levels of calcium on skin CaBP

Groups	Skin CaBP (μg/mg protein)	Serum calcium (mg/100 ml)
Expt I		
D+ Ca+	27.1 ± 2.2 (11)	10.3 ± 0.1 (9)
D− Ca+	15.9 ± 1.2[b] (6)	8.0 ± 0.3[b] (6)
Expt II		
D− Ca−	34.1 ± 3.1 (8)	5.5 ± 0.1 (7)
D+ Ca−	51.5 ± 3.8[a] (9)	7.1 ± 0.2[b] (9)

Number in brackets indicates no. animals: [a] $p < 0.01$; [b] $p < 0.001$

In expt I weanling rats were raised for 4 weeks on a diet normal in calcium and vitamin D (D+ Ca+ rats) or a diet normal in calcium and without vitamin D (D− Ca− rats). In expt II the weanling rats were raised on a diet containing 0.02% of calcium and no vitamin D. After 4 weeks, the rats were given 1 μg vitamin D_3 orally (D+ Ca− rats) or its solvent (D− Ca− rats) for 3 days

the skin (34.1 ± 3.1 μg/mg total protein; table 1). However, when these rats were given vitamin D for 3 days skin CaBP increased significantly (51.5 ± 3.8 μg/mg total protein, $p < 0.01$). This value is also significantly ($p < 0.01$) different from the value found in normal controls.

4. Discussion

This data shows that rat skin contains a protein with calcium-binding activity. The molecular weight of this CaBP (9000 ± 2000) its ionic properties on ion-exchange chromatography, and its electrophoretic pattern in the presence of EDTA are similar to those of rat intestinal CaBP. Comparison of the amounts of skin CaBP obtained in a 3000 × *g* and in a 100 000 × *g* supernatant seems to indicate that the cellular localization of this protein is mainly cytosolic. In spite of physical similarities no immunological similarity could be found between rat skin and intestinal CaBPs. Skin CaBP did not crossreact with antiserum raised against intestinal CaBP. Conversely cross-reaction was not observed between intestinal CaBP and antiserum raised against skin CaBP. Clarification of this difference in immunologic properties must await further studies on the amino acid sequence and conformational characteristics of these two CaBPs.

Another significant difference between rat skin and intestinal CaBPs is the apparent continued synthesis of the skin protein in the absence of dietary vitamin D. Nevertheless, the amount of CaBP in the skin of vitamin D-deficient rats is significantly lower indicating that the CaBP activity detected in rat skin is indeed dependent on the vitamin D status.

It is well documented that the level of intestinal vitamin D-dependent CaBP is regulated in an adaptative way to reflect the level of calcium present in the diet [17,26]. Our results would suggest that this is also the case with skin CaBP since low dietary calcium prevents the decrease in CaBP level observed in the absence of vitamin D. However, when the vitamin D and calcium deprived rats are given vitamin D for 3 days the amount of skin CaBP increases significantly ($p < 0.01$) and reaches values higher than in the normal animals (table 1). This data would indicate that:

(i) Vitamin D, or more likely its active metabolite 1,25-dihydroxyvitamin D, stimulates the synthesis of skin CaBP;
(ii) As shown for intestine and bone CaBPs [17,26], the amount of CaBP in rat skin adapts inversely to the level of dietary calcium, most probably through changes in extra-cellular calcium concentration.

As for the persistence of a decreased yet still considerable amount of CaBP in rat skin after 4 weeks of vitamin D deprivation this may be a reflection of the persistence of some vitamin D in these animals and/or of the slower cell turnover rate of skin [26,27] as compared to the intestinal mucosa. A greater residence time of CaBP in chick kidney than in intestine has also been found and related to the slower cell turnover rate of kidney tissue [28,29].

The role of skin CaBP can not be elucidated here. However, the presence of a vitamin D-dependent CaBP in a tissue which is the unique site of endogenous vitamin D synthesis, may suggest a possible physiological role of this protein in vitamin D and/or calcium metabolism in the skin.

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